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Research paper

Rapid characterization of binding specificity and cross-reactivity of antibodies using recombinant human protein arrays

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ABSTRACT

Antibodies are routinely used as research tools, in diagnostic assays and increasingly as therapeutics. Ideally, these applications require antibodies with high sensitivity and specificity; however, many commercially available antibodies are limited in their use as they cross-react with non-related proteins. Here we describe a novel method to characterize antibody specificity. Six commercially available monoclonal and polyclonal antibodies were screened on high-density protein arrays comprising of ~10,000 recombinant human proteins (Imagenes). Two of the six antibodies examined; anti-pICln and anti-GAPDH, bound exclusively to their target antigen and showed no cross-reactivity with non-related proteins. However, four of the antibodies, anti-HSP90, anti-HSA, anti-bFGF and anti-Ro52, showed strong cross-reactivity with other proteins on the array. Antibody–antigen interactions were readily confirmed using Western immunoblotting. In addition, the redundant nature of the protein array used, enabled us to define the epitopic region within HSP90 of the anti-HSP90 antibody, and identify possible shared epitopes in cross-reacting proteins.

In conclusion, high-density protein array technology is a fast and effective means for determining the specificity of antibodies and can be used to further improve the accuracy of antibody applications.

1. Introduction

Since antibodies possess unique antigen binding sites, which can discriminate between millions of different antigens, their specificity is the basis for their use in many applications. Antibodies are routinely used as tools in teasing apart molecular pathways in techniques such as Western immunoblotting and ELISA. The diagnosis of disease and targeted therapies are increasingly becoming more dependent upon antibodies (Waldmann and Morris, 2006). More recently, antibody arrays, containing hundreds or thousands of antibodies, have been shown to be invaluable in protein profiling (Barry and Soloviev, 2004). To exploit the full potential of these applications, antibodies with high specificity are required and potential cross-reactivities need to be identified. Although commercial antibodies are widely used within the research field, many lack the specificity required to be used as reliable tools mainly because there are few robust methods available to assess non-specific binding.

New developments in immunology are implementing array technology to assess antibody function (Murphy and Cahill, 2005). Peptide arrays, random peptide libraries (Reineke et al., 2002; Poetz et al., 2005; Yu et al., 2006; Paley et al., 2007) multiplexed bead approaches (Warren and Bettadapura, 2005; Schwenk et al., 2007) are increasingly being used to identify the interactions of antibodies. While often highly successful, these methods are limited by the low

Abbreviations: ELISA, Enzyme-Linked ImmunoSorbent Assay;GAPDH, glyceraldehyde 3-phosphate dehydrogenase;HSP-90, heat shock protein 90;HSA, human serum albumin;bFGF, basic fibroblast growth factor;ICln, chloride channel, nucleotide-sensitive, 1A.

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number of potential antigens or require lengthy experimental procedures. Protein microarrays containing hundreds of proteins have been successfully applied to examine specificity and cross-reactivity of custom-made antibodies to yeast (Michaud et al., 2003) and human liver proteins (Hu et al., 2007). Several studies have demonstrated the potential of protein arrays in the identification of antigen–antibody interactions for human proteins (Bussow et al., 1998; Holt et al., 2000). The large protein arrays used represent a powerful alternative approach for identifying potential cross-reactivities in a high-throughput format, as more than 10,000 recombinant human proteins can be simultaneously analyzed. In addition, the sequence information for each expressed protein is available (Imagenes), allowing rapid identification of binding interactions.

In this study, we describe a rapid approach to define the molecular targets for various commercial monoclonal and polyclonal antibodies using high-density protein arrays. We screened six antibodies commonly used in different fields of research to determine possible binding interactions on the protein array. Our results confirm that some monoclonal antibodies are mono-specific, however others show strong cross-reactivity. Since the sequence information is available for all cross-reacting proteins, we were able to map epitopic regions shared between these antigens. In conclusion, high-density protein arrays are a powerful high-throughput technique to determine cross-reactivity of antibodies which may help to assess the quality of newly produced antibodies and confirm the specificity of antibodies already in use.

2. Materials and methods

2.1. Protein arrays

The high-density protein arrays used in this study contain 38,016 *Escherichia coli* expression clones from a human foetal brain cDNA (hEx1) library arrayed on PVDF membranes (Imagenes, GmbH). The expression clones were generated in the expression vector pQE30NST (GenBank Accession No. AF074376) and were transformed into *E. coli* strain SCS1 (Stratagene) (Bussow et al., 1998; Horn et al., 2006). Sequence information for individual clones recognized by antibodies was provided by Imagenes GmbH.

2.2. Antibody screening

Prior to antibody screening, the dried colonies were removed from the PVDF membranes with tissue paper and TBSTT (TBS supplemented with 0.05% Tween 20 and 0.5% TritonX-100). Followed by three washes, for 20 min each, in TBST (TBS supplemented with 0.05% Tween 20) and blocking with 3% (w/v) skimmed milk powder (Marvel) in TBST for 3 h at room temperature, the membranes were incubated overnight with one of the following primary antibodies: anti-HSP90 monoclonal antibody, (sc-13119; Santa Cruz Biotechnology); anti-GAPDH monoclonal antibody (Clone 6C5; Research Diagnostics); monoclonal anti-HSA (Clone 1C8, Hytest), monoclonal anti-plCln (Clone 32; BD Biosciences); monoclonal anti-basic Fibroblast Growth Factor (bFGF), (Clone FB-8; Sigma); polyclonal anti-Ro52 (sc-20960, Santa Cruz Biotechnology). All antibodies utilised in this study are human specific and were used at dilution (1/1000, in 2% BSA/TBST) recommended by the manufacturer. Primary antibody incubation was followed by three washes of 20 min each in TBST. The protein arrays were then incubated with the secondary antibody conjugated with alkaline phosphatase (AP); anti-mouse IgG (A1418; Sigma,) or anti-rabbit IgG (111-055-045; Jackson ImmunoResearch) appropriate to each primary antibody for 1.5 h. Following three washes for 20 min each in TBST, the membranes were incubated for 10 min in AP buffer (1 mM $MgCl_2$, 100 mM Tris–Cl, pH 9.5) and 5 min in 0.125 mM Attophos in AP buffer. Filters were illuminated with long-wave UV light (460 nm EPI) and images were taken using a high resolution CCD camera (Fuji LAS3000). Image analysis was performed using VisualGrid (GPC Biotech). In order to eliminate any background binding, each secondary antibody used for detection was additionally screened against the arrays in the absence of the primary antibody and all expression clones detected were removed from the list generated for the primary antibody. The identities of clones bound by tested primary antibodies were obtained from Imagenes GmbH and confirmed by sequencing.

2.3. Recombinant protein expression and purification

The *E. coli* clones expressing the recombinant HIS-tagged proteins from the hEx1 library were purchased from Imagenes GmbH. Clones were grown in 5 ml liquid cultures and protein expression induced with 1mM IPTG and purified as previously described using nickel affinity chromatography (Bussow et al., 1998; Lueking et al., 1999). The size of each purified protein was determined by SDS PAGE using Coomassie staining and Western immunoblotting using a mouse anti-RGS–His antibody (34650, Qiagen).

2.4. Western immunoblotting

The purified proteins were separated by SDS PAGE and transferred onto PVDF membranes at 100 V for 1 1/2 h. The membrane was blocked for 2 h in 3% Marvel/TBST, washed in TBS (3×10 min) and incubated for 2 h with the primary antibody as described above. Antibody binding was detected using an alkaline phosphatase-conjugated anti-mouse, or anti-rabbit, IgG secondary antibody (Sigma) and visualized on a Fuji LAS 3000 imager.

2.5. Epitope mapping

Amino acid sequence alignment and definition of potential epitope regions were performed using the web-based software SIM (http://us.expasy.org/tools/sim-prot.html), LALIGN (http://www.ch.embnet.org/software/LALIGN_form.html) and CLUSTALW.

3. Results

3.1. Characterization of commercial antibodies

Probing protein arrays with antibodies enables the assessment of specificity and cross-reactivity on large numbers of potential antigens in parallel. Here we

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Table 1
Antibody–antigen interactions identified using high-density protein arrays and confirmed by Western immunoblotting

Antibody	Proteins bound by antibody	Acc. number
α-GAPDH	GAPDH	P04406
α-ICln	Chloride channel, nucleotide sensitive 1a	P54105
α-bFGF	bFGF	P78443
	Pyrroline-5-carboxylate reductase 1	P32322
	Alu7	P39194
	Heterogeneous nuclear ribonucleoprotein D-like	Q6SPF2
	CDC42 binding protein kinase beta	NP_006026
α-HSA	Human serum albumin	P02768
	Phosphatidylethanolamine-binding protein	P30086
	ADP-ribosylation factor-like protein 5A.	Q9Y689
	Kv channel-interacting protein 1 (KChIP1)	Q9NZI2
α-Ro52	Ro 52	NM_003141
	GPAA1P anchor attachment protein 1	NM_003801
	Zinc finger protein 6	NM_021998
	Zinc finger protein 354A	NM_005649
	HLA-B associated transcript 3	NM_080702
	Triosephosphate isomerase 1	NM_000365
	Cell division cycle associated 5	NM_080668
	chromosome 10 open reading frame 104	NM_173473
	Hypothetical protein MGC2803	BC000216
	CRAMP1L , Crm, cramped-like	AB037847
	Hippocalcin	BC001777
	Hypothetical protein LOC51031	NM_016080
	cDNA DKFZp761G1421	AL713634
α-HSP90	HSP90	P08238
	SH2-B gamma signalling protein	AF227969
	Radixin	L02320

characterize the specificity and cross-reactivity of a panel of commonly used commercially available antibodies. These were the monoclonal antibodies: anti-HSP90, anti-HSA, anti-pICln, anti-GAPDH, anti-bFGF and a polyclonal anti-Ro52 antibody. We screened these antibodies against the hEx1 protein arrays (Bussow et al., 1998, 2000) which contain a redundant set of 38,016 clones expressing recombinant human proteins. The antibodies characterized here and the corresponding antigens identified in this study are listed in Table 1.

3.2. Screening protein arrays confirms mono-specificity of two antibodies

Two of the six antibodies tested on the protein arrays demonstrated mono-specificity: anti-pICln and anti-GAPDH, both of which are monoclonal. The anti-GAPDH antibody (Clone 6C5) is widely reported as being highly specific with no other banding in Westerns reported (e.g. (Li et al., 2004)). This antibody recognized 81 expression clones on the arrays, all of which express GAPDH protein. A representative section of the protein array is shown in Fig. 1a. Sequencing revealed that all clones express full length protein. The antibody–GAPDH binding was confirmed using Western immunoblotting (Fig. 1b). The monoclonal anti-pICln (Clone 32) recognized 6 expression clones on the hEx1 protein array (Fig. 1c). Sequencing confirmed all clones expressed large fragments or full-length pICln. All 6 pICln clones identified in this study express a pICln fragment which includes the immunogenic region (92–201 amino acids) as reported by the manufacturer. The antibody–pICln binding was confirmed for all 6 clones using Western immunoblotting (Fig. 1d). The banding pattern in the Westerns matches our previously reporting that this antibody recognises both the full length protein and degradation products both in human platelet lysates and for bacterially expressed recombinant protein (Larkin et al., 2004). Mass

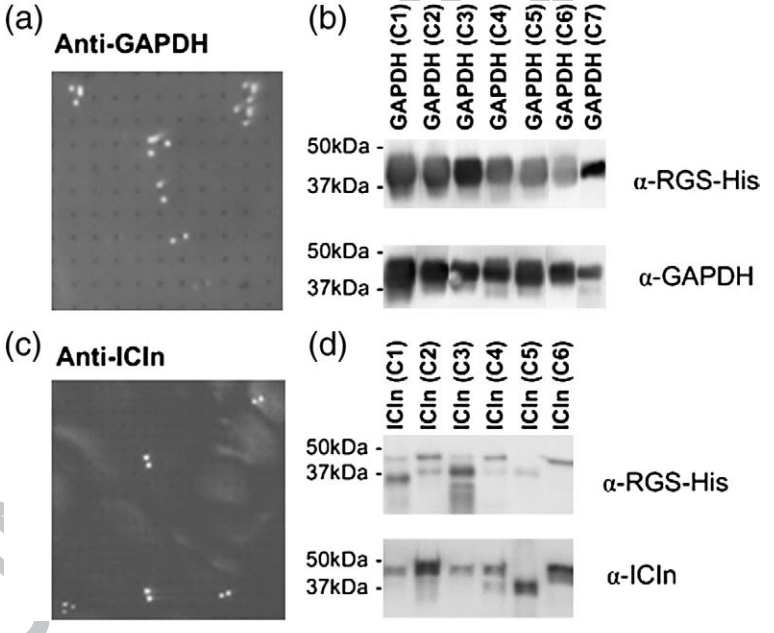

Fig. 1. Mono-specific antibodies verified by high-density protein arrays. (a) Representative section of the protein array probed with monoclonal anti-GAPDH antibody, all identified clones express full-length GAPDH protein. (b) Western immunoblot analysis of recombinant RGS-His tagged GAPDH confirms presence of GAPDH (α-RGS-His) and its interaction with the α-GAPDH antibody. (c) Protein array probed with monoclonal anti-ICln antibody. (d) Six clones expressing different ICln fragments were identified and confirmed using Western immunoblot.

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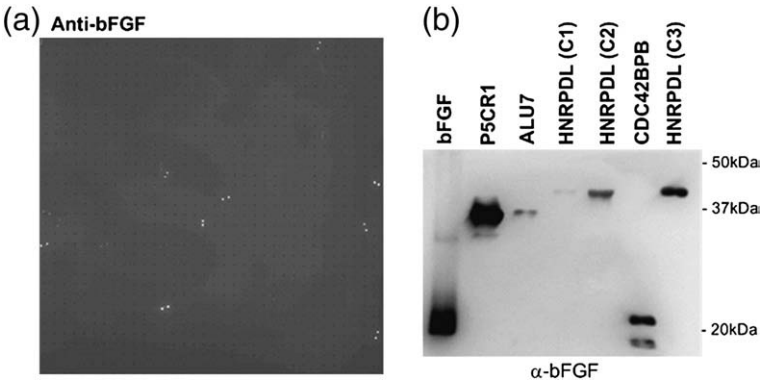

Fig. 2. Cross reactive binding identified by high-density protein array screening. (a) Representative section of the protein array probed with monoclonal anti-bFGF antibody, screening of the arrays with anti-bFGF revealed the antibody binds 4 proteins other than bFGF. (b) Western immunoblot analysis with anti-bFGF antibody confirms binding to bFGF, Pyrroline-5-carboxylate reductase (P5CR1), ALU7 CDC42 binding protein kinase beta (CDC42BPB) and three clones (C1–C3) expressing Heterogeneous nuclear ribonucleoprotein D-like/CArG binding factor (HNRPDL).

spectrometry has since confirmed the identity of the putative degradation bands as ICln (Niamh Moran and Achim Treumann, personal communication). Clone C4 codes for and expresses a shortened ICln protein, missing the first 67 amino acids.

3.3. Protein arrays identify cross-reactivities for monoclonal and polyclonal antibodies

Monoclonal antibodies against HSA and bFGF antibody revealed a number of cross-reactivities upon screening of the hEx1 protein array. We observed and confirmed that anti-bFGF (FB-8) cross-reacted with 4 other proteins on the array and in Western immunoblots, including three clones expressing Heterogeneous nuclear ribonucleoprotein D-like/CArG binding factor as shown in Fig. 2. The antibody used has previously been reported to show numerous non-FGF bands in Western immunoblots of lysates from a large variety of lymphoma cell lines (Krejci et al., 2003), including bands matching in size the proteins detected on the protein arrays. The monoclonal anti-HSA (1C8) antibody, which is recommended for ELISA use and explicitly not recommended for Western immunoblotting by the manufacturer, demonstrated besides specificity to the cognate HSA antigen, cross-reactivity with three unrelated proteins on the protein arrays and in Western immunoblot (Table 1).

Next, we tested a polyclonal anti-Ro52 antibody for specificity. Polyclonal antibodies represent a mixture of immunoglobulin molecules raised against an antigen, each capable of recognizing a different epitope. It is therefore likely that polyclonal antibodies have higher cross-reactivity. Using protein arrays we analyzed the anti-Ro52 antibody (sc-20960) raised against amino acids 141–280 of the Ro52 of human origin. The screening revealed binding of the antibody to 18 clones expressing 13 different proteins, including the cognate Ro52 (Table 1), which was the highest number of cross-reacting proteins for any antibody tested.

3.4. Identification of shared epitopes between unrelated proteins

The monoclonal anti-HSP90 (sc-13119) antibody is shown by the manufacturer to cross-react with at least two other proteins when used to detect HSP90 in cell lines. The antibody bound 25 clones on the protein array (Fig. 3a). 22 of these clones express HSP90 protein and 3 clones express non-cognate proteins. Confirmation of these interactions was performed via Western immunoblotting (Fig. 3b). Included in the Western immunoblot are the clones expressing the longest and shortest fragments of HSP90 detected by the antibody, confirming that the location of the epitope is near the carboxyl terminal (610–725 amino acids) as reported by the manufacturer (Santa Cruz Biotechnology Inc.). The binding of the anti-HSP90 antibody to the non-cognate proteins SH2-B and Radixin was also confirmed via Western immunoblotting (Fig. 3b). Sequencing of the third non-cognate cross-reacting clone revealed the clone to contain a cDNA for 2,4-dienoyl-CoA reductase. However, sequencing also revealed that the encoding protein is not in frame with the 5′ RGS-His tag. This not only makes rapid purification of the encoding protein difficult but also raises the strong possibility that the antibody is cross-reacting with the in-frame nonsense protein.

In order to identify potential shared epitopes, the amino acid sequences of SH2-B and Radixin were subjected to pairwise alignment (LALIGN and SIM) against the HSP90 epitope region sequence. One region of similarity could be identified as a shared epitope (Fig. 3c).

4. Discussion

In this paper, we demonstrate the use of protein/antigen arrays for the routine characterization of antibodies used in basic research as well as, potentially, in diagnostics. Each antibody can be readily screened against an appropriate protein expression library, in this case a human foetal brain library arrayed on PVDF membranes (Imagenes, GmbH). Unlike previous approaches to characterize antibodies, which could at best identify potential epitopes of a particular antibody, this approach identifies the actual target protein. In addition, potential cross-reacting proteins can also be identified using this method, which is important information for assessing an antibody, particularly for *in vivo* studies as well as diagnostics or therapeutics.

One clear advantage of this approach is that expression clones bound by the primary antibodies can readily be

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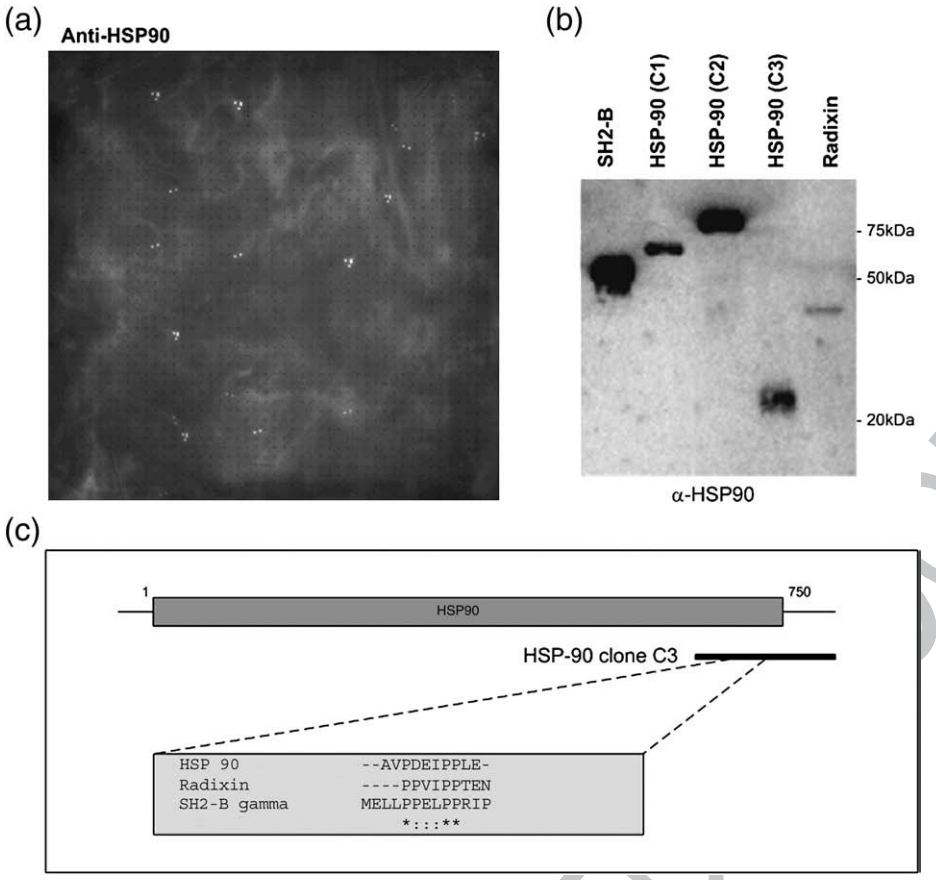

Fig. 3. Protein array analysis identifies shared epitopes between unrelated proteins. (a) Representative section of the protein array probed with monoclonal anti-HSP90 antibody. Anti-HSP90 antibody recognizes 23 clones, 2 of which express a protein other than HSP90. (b) The cross-reactivities of anti-HSP90 were confirmed using Western immunoblot of the purified recombinant protein. Lane 1: SH2-B gamma signalling protein (SH2-B); three: different HSP90 clones; Radixin. (c). Using the redundancy of the library, we could define the region of the epitope within HSP90 and use this data to find potential epitopes within the cross-reacting proteins.

retrieved from the original library and large quantities of the recombinant protein produced for further experimental analyses. In this case, His-tagged recombinant human proteins were purified using chelate-affinity chromatography and western immunoblotting allowed confirmation of antibody interactions observed on the array. Further, most clones used in these arrays have sequence information available. Such information confirms the protein identity and can be used to gather information on putative epitopes shared between cognate antigens and non-cognate proteins showing cross-reactivity with the tested antibody. In one example, the anti-HSP90 antibody cross-reacted with a number of clones expressing proteins of the HSP90 protein, including one clone encoding only a relatively short stretch of the HSP90 C-terminus. This permitted a quick identification of a short epitope region within the cognate protein.

The interactions of the antibody with cross-reactive proteins can be deduced by alignment of the primary amino acid sequences of the antigens and cross-reactive proteins. However, it would not be possible to predict these interactions *a priori*. The cross-reactivity between two antigens may occur when an antibody recognises the same epitope in two different proteins. As demonstrated in this study in the case of the HSP90, mapping of the epitope is possible when cross-reactivity occurs between two different proteins with the same epitope. However, no common epitopes were found upon amino acid sequence comparison in three of four investigated antibodies showing cross-reactivity with unrelated proteins. Cross-reactive binding due to the ability of an antibody to recognise different unrelated epitopes on separate antigens was previously described (Berzofsky and Schechter, 1981; James et al., 2003). Such binding may be caused by discontinuous epitopes, separated by several amino acids not directly contributing to the antibody binding (Poetz et al., 2005) or multi-specific antibodies (Bhattacharjee and Glaudemans, 1978; Van Regenmortel, 1998) making it impossible to map epitopes by simple sequence comparison.

Perhaps the most significant potential application for protein array screens is during antibody production to determine degree of specificity and identify cross-reacting proteins. Such information could prove invaluable for the scientist in deciding which antibody is best suited for a particular task. This application could prove even more valuable in the field of diagnostic antibodies.

Overall, our results point towards a future central role to be played by protein arrays in the characterization of

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antibodies, including their integration within the production process. The protein array technology described in this study combines the simplicity and speed of standard ELISA procedures with the parallel assessment of thousands of different human proteins for specificity and cross-reactivity with the investigated antibody. With the increased availability of higher density microarrays containing not only proteins with post-translational modifications but also other potential antigenic targets such as lipids and nucleic acids, the approach demonstrated in this study should become an attractive option for the quick analysis of antibodies.

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